(12) UK Patent Application (19) GB (11) 2 215 335 (13) A

(43) Date of A publication 20.09.1989

(21) Application No 8805265.9

(22) Date of filing 04.03.1988

(71) Applicant -
Shell Internationale Research Maatschappij B.V

(Incorporated in the Netherlands)

Carel van Bylandtlaan 30, The Hague, Netherlands

(72) Inventors
Andrew Williams
Robert John Pryce

(74) Agent and/or Address for Service
K R I Hunter
4 York Road, London, SE1 7NA, United Kingdom

(51) INT CL⁴
C12N 11/08 11/06

(52) UK CL (Edition J)
C3H HH1 H200 H241 H242

(56) Documents cited
None

(58) Field of search
UK CL (Edition J) C3H HH1
INT CL⁴ C12N
DIALOG ONLINE SEARCH (BIOTECH, WPI)

(54) Microgel immobilised enzymes

(57) An immobilized enzyme conjugate soluble or dispersible in an organic solvent-containing medium (e.g. methanol or tetrahydrofuran) comprises a polymer microgel which has been prepared from at least one monomer, the or one of the monomers having been selected so as to introduce hydrophobic groups into the microgel, the microgel being conjugated via covalent linkages with the enzyme. The polymer may be alkoxyalkyl acrylate or methacrylate, to which the enzyme (e.g. α-chymotrypsin, esterase or sulphatase) may be bound by carbodimide linkages.

GB 2 215 335 A

IMPROVEMENTS RELATING TO MICROGEL IMMOBILISED ENZYMES

This invention relates to immobilised enzyme conjugates, their preparation and their use in enzyme catalysed reactions.

The immobilisation of enzymes on supports to enable their catalytic activity to be more readily exploited is well known. Such supports include polymer microgels to yield microgel-enzyme conjugates which are soluble or dispersible in the aqueous media in which enzyme catalysed reactions are often carried out. However, not infrequently, it is desired to carry out an enzyme catalysed reaction in an organic medium. While this was originally considered not to be possible, more recently techniques have been devised which involve modification of the enzyme itself by introduction of groups to convey solubility and/or dispersibility in organic solvents. This can adversely affect the reactivity of the active sites of the enzyme. Precipitation of enzymes on glass powder has also been proposed (Grunwald et al, JACS, 108, 6732, (1986)), but such glass powder immobilised enzymes have also been found to have poor reactivity.

The present invention seeks to provide an immobilised enzyme conjugate which can be

satisfactorily employed in organic solvent-containing media without a substantial loss of activity of the enzyme.

According to the invention, we provide an immobilised enzyme conjugate soluble or dispersible in an organic solvent-containing medium, comprising a polymer microgel which has been prepared from at least one monomer, the or one of the momomers having been selected so as to introduce hydrophobic groups into the microgel, the microgel being conjugated via covalent linkages with the enzyme.

The polymer microgel is preferably an acrylate and/or methacrylate polymer comprising at least one acrylate or methacrylate monomer containing a hydrophobic substituent such as an alkoxy group, such as an alkoxyalkyl acrylate or methacrylate, for example 2-ethoxyethyl methacrylate. Other hydrophobic substituents may be present instead of or in addition to the alkoxy group, such as aryl substituents. The nature of the hydrophobic substituent or substituents depends on the organic medium in which it is wished to employ the immobilised enzyme. Other comonomers are for example selected from methyl methacrylate, ethylene glycol dimethacrylate and acrylic acid. A preferred polymer is derived from a monomer composition comprising, by molar percent, up to 60% ethoxyethyl methacrylate (preferably 25 to 35%, especially about 30%), from 5 to 20% of ethylene glycol dimethacrylate (preferably 5 to 15%, especially about 10%), from 5 to 20% acrylic acid (preferably 10 to 20%, especially about 15%) and the balance of methyl methacrylate. Alternative microgels include polystyrene and

polydivinylbenzene microgels having reactive side chains capable of covalently linking with the enzyme.

The enzyme may be any enzyme which it is desired to employ in an enzyme catalysed reaction in an organic solvent-containing medium. Examples of such enzymes are α-chymotrypsin for use in the hydrolysis of esters, such as N-benzoyl-L-tyrosine ethyl ester, sulphatase for use in the degradation of organic sulphates such as p-nitrophenyl sulphate, and esterase for use in the degradation of esters such as p-nitrophenyl acetate and p-nitrophenyl butyrate.

The enzyme and microgel may be covalently linked by any known coupling method, such as by using a carbodiimide which reacts with the carboxylic groups of the polymer and with suitable active sites on the enzyme. Alternative coupling methods may be used for example where the polymer contains amino, sulphonyl chloride or diazo groups which can link respectively with carboxyl, amino or aryl groups on the enzyme.

The polymer microgel may be prepared by conventional emulsion polymerisation using methods such as those described by Weatherhead et al, Makromol. Chemie, 181, 2529, 1980, Stacey et al, Makromol. Chemie, 181, 2517, 1980 and Hopkins et al, J.C.G. Perkin Trans. II, 891, 1983.

It has been found that the immobilised enzyme conjugates of the present invention offer a number of advantages. Thus they enable enzyme catalysed reactions to be carried out in organic solvents without substantial loss of catalytic activity. The dispersibility of the immobilised enzyme is high, giving improved enzyme usage and mass transfer when compared with the use of non-immobilised bulk enzymes. Microfiltration techniques can readily be

employed to aid recovery of the immobilised enzymes after use, thus allowing recycle and reuse of the catalyst and also reducing the demand for product stream purification. These advantages make the hydrophobic microgel immobilised enzymes especially suitable for use in the production of, for example, fine and speciality chemicals, e.g. agrochemicals or pharmaceutical chemicals, and intermediates therefor, many of which are likely to be water insoluble. The invention offers the possiblity of transferring the enzyme, in a dispersed form, into an organic solvent, or water-solvent system, to allow the enzyme to act on such water insoluble materials.

The invention therefore also includes the use of the enzyme conjugates of the invention in enzyme catalysed reaction in organic solvent-containing media.

The following examples illustrate the invention.

Example 1 Preparation of Polymer Microgel

A monomer feed was prepared having the following composition (molar per cent):

methyl methacrylate	45%
2-ethoxyethyl methacrylate	30%
ethylene glycol dimethacryate	10%
acrylic acid	15%

The monomer feed (5ml) was added to water (100ml) with sodium dodecysulphate (100mg) in a glass soda-bottle which was equilibrated to 70°C in an oil bath on a heater-stirrer plate. The mixture was purged with nitrogen, emulsion polymerisation initiated with ammonium persulphate (50mg) and the bottled sealed. The polymerisation was stopped with quinol (50mg) after the characteristic 'blue'

opalescence had appeared but prior to formation of solid aggregates. The solution was centrifuged to remove any coagulated matter and purified by repeated precipitation of the polymer with dilute acid, centrifugation and solution by the addition of dilute sodium hydroxide. The sample was purged of residual surfactant by stirring with strong base macroreticular anion exchange resin (Amberlyst A26) in its hydroxide form. In all cases the product polymer was tested for purity by passage through an analytical Sephadex G25 column. A yield approaching 100% was obtained.

The prepared polymer was essentially mono-disperse with a diameter of 441 Å in the dry state. The wet diameter of the polymer was found to be 918 Å in pH 7.42 phosphate buffer, 1075 Å in pH 5.8 acetate buffer and 1584 Å in methanol. The molecular weight of the polymer bead (excluding water) may be calculated from the dry diameter assuming a reasonable density of unity to be 2.70×10^7.

Hydrogen ion titration showed that the microgel possessed 4.53×10^{-7} moles of equivalent acid per mg of solid. Based on a 15% molar feed composition there is 36% incorporation of the acrylic acid into the microgel, and 1.22×10^4 carboxylate groups per polymer bead at a density of one carboxylate per 50 $Å^2$ of dry particle surface and per 217 $Å^2$ wet particle surface.

Example 2 Preparation of Polymer Microgel-Enzyme Conjugate

Conjugates with the polymer microgel prepared as in Example 1 were prepared of the following commercially available enzymes:

- α-chymotrypsin
- sulphatase
- esterase

The conjugates were prepared as follows:

Enzyme (5-25mg in 5ml water) was mixed with polymer (50mg in 5ml water). 1-ethyl-3-(3'-N, N-dimethylaminopropyl)carbodiimide HCl (EDC) (5mg) was added and the mixture stirred overnight at 4°C (pH 7.1-7.2). No ionic complex formation was observed when the enzyme and polymer solutions were mixed. The urea by-product and unbound enzyme were removed by ultra-filtration on Amicon filters with appropriate molecular weight cut-off (30 000 or 300 000). Integrity of the enzyme-polymer conjugate was demonstrated on an analytical Sephadex G25 column. The α-chymotrypsin conjugate had 405 active sites of enzyme per polymer particle giving corresponding densities of one α-chymotrypsin molecule per 1.50 X 10^3 A^2 and per 6.54 x 10^3 A^2. About 29% of the wet microgel surface is covered by this enzyme as judged from the dimensions of α-chymotrypsin.

The α-chymotrypsin-polymer conjugate had a dry diameter of 588 A and wet diameters of 1455 and 1102 A in phosphate and acetate buffers respectively. The sulphatase and esterase polymer conjugates were formed to be somewhat similar in physical characteristics.

The yield in the coupling of the various enzymes to the polymer using EDC was >90% as determined by

activity tests on the effluents which passed the ultra-filtration membranes. Tests were carried out to ensure that polymer was retained and also to show that the native enzymes were passed by the ultra filters employed. Chromatographic analyses indicated single peaks for the enzyme-polymer conjugate, which was soluble in both water and methanol.

Example 3 Hydrolysis of N-carbobenzoxy-L-tyrosine-p-nitrophenyl ester using α -chymotrypsin polymer conjugate

The α -chymotrypsin polymer conjugate prepared as given in Example 2 (0.25-1 mg) in a solvent of water (0.025-0.1 ml), was added on the tip of a glass rod to a 1cm reaction cell, thermostatically controlled at 25°C containing N-carbobenzoxy-L-tyrosine-p-nitrophenyl ester (0.016-50 mg) in 2.5 ml of the solvent systems given in Table 1.

Hydrolysis to give p-nitrophenol was confirmed by spectroscopy and the reaction was followed by ultra violet spectrophotometry at 335nm and the rate of reaction determined. The Michaelin-Menten parameters K_m (affinity constant) and k_{cat} (activity constant) were determined by Lineweaver-Burk analysis.

The results are given in Table 1 (M = moles per litre; I = ionic strength; s = seconds):

Table 1

Solvent	$[H_2O]$/M	(% v/v)	K_m/M	$k_{cat}.s^{-1}$
MeOH	0.917	(1.65)	1.14×10^{-4}	2.58×10^{-2}
	1.834	(3.30)	5.28×10^{-4}	4.81×10^{-2}
	2.751	(4.96)	1.10×10^{-3}	0.152
	4.585	(8.26)	7.03×10^{-3}	1.48
THF	2.22	(4.0)	4.85×10^{-4}	7.11×10^{-2}
	2.22*1.	(4.0)	$>5 \times 10^{-3}$	<0.21
	4.44	(8.0)	9.11×10^{-4}	0.125

*1. Water content made up with acetate buffer, pH 5.8, I = 0.05M rather than double distilled water.

Example 4 Degradation of p-nitrophenyl sulphate using polymer-bound sulphatase

Sulphatase polymer conjugate prepared as described in Example 2 (0.25-1 mg) in a solvent of water (0.025-0.1 ml) was added on the tip of a glass rod to a 1cm reaction cell, thermostatically controlled at 25°C containing p-nitrophenyl sulphate (0.002-0.21 mg) in 2.5 ml of the solvent system given in Table 2. The concentration of sulphatase was 2.5 X 10^{-4} g/ml.

Degradation was followed by ultra violet spectrophotometry at 400nm. From the determined rate of reaction, the Michaelin-Menten parameters K_m and k_{cat} were determined by Lineweaver Burk analysis. The results are given in Table 2 and compared with a control experiment using unbound "native" sulphatase.

Table 2

Enzyme	Solvent	$[H_2O]$/M	K_m/M	k_{cat}/M.5^{-1}
native sulphatase	acetate buffer pH 5.8, I = 0.05M	-	2.5×10^{-5}	2.69×10^{-7}
polymer-bound sulphatase	acetate buffer pH 5.8, I = 0.05M	-	4.03×10^{-5}	1.42×10^{-7}
polymer-bound sulphatase	MeOH	1.99	5.59×10^{-5}	4.13×10^{-7}

It will be seen that the affinity constant K_m is not adversely affected by reaction in organic medium and that the activity constant is actually increased when working in the organic medium.

Example 5. Degradation of p-nitrophenyl esters using polymer-bound esterase

Esterase polymer conjugate prepared as described in Example 2 (0.25-1.0 mg) in water (0.025-0.1 ml) was added on the tip of a glass rod to a 1cm reaction cell, thermostatically controlled at 25°C containing p-nitrophenyl acetate or butyrate (0.004-1.7 mg) in 2.5 ml of the solvent system given in Table 3. The concentration of esterase was 6.25 X 10^{-5}g/ml.

Degradation was followed by ultra violet spectrophotometry at 400nm. The Michaelin-Menten parameters were determined by Lineweaver Burk analysis. The results are given in Table 3 and compared with a control experiment using unbound "native" esterase.

Table 3

Enzyme	Substrate	Solvent	$[H_2O]$/M	Km/M	k_{cat}/M.s^{-1}
native esterase	p-nitrophenyl acetate	phosphate buffer pH 7.42, I = 0.1M	-	3.10×10^{-4}	7.04×10^{-6}
polymer bound esterase	p-nitrophenyl acetate	phosphate buffer pH 7.42, I = 0.1M	-	6.28×10^{-4}	7.84×10^{-6}
polymer bound esterase	p-nitrophenyl acetate	MeOH	0.917	2.54×10^{-4}	3.0×10^{-7}
polymer bound esterase	p-nitrophenyl butyrate	MeOH	0.917	7.67×10^{-5}	3.41×10^{-8}

CLAIMS

1. An immobilised enzyme conjugate soluble or dispersible in an organic solvent-containing medium, comprising a polymer microgel which has been prepared from at least one monomer, the or one of the momomers having been selected so as to introduce hydrophobic groups into the microgel, the microgel being conjugated via covalent linkages with the enzyme.
2. A conjugate according to claim 1 wherein the polymer microgel is an acrylate and/or methacrylate polymer.
3. A conjugate according to claim 2 wherein the polymer comprises an alkoxyalkyl acrylate or methacrylate.
4. A conjugate according to claim 3 wherein the polymer is derived from a monomer composition comprising, by molar per cent, up to 60% ethoxyethyl methacrylate, 5 to 20% ethylene glycol dimethacrylate, 5 to 20% acrylic acid and the balance of methyl methacryate.
5. A conjugate according to any one of the preceding claims wherein the microgel and enzyme are bound via carbodiimide linkages.

6. A conjugate according to any one of the preceding claims wherein the enzyme is selected from ∝-chymotrypsin, esterase and sulphatase.

7. A method of carrying out an enzyme-catalysed reaction comprising contacting a substrate with a conjugate according to any one of the preceding claims in an organic solvent-containing medium.

8. A method according to claim 7 wherein the solvent comprises methanol or tetrahydrofuran.

9. A method according to claim 7 or 8 wherein the enzyme is ∝-chymotrypsin and the substrate is an ester.

10. A method according to claim 7 or 8 wherein the enzyme is sulphatase and the substrate is an organic sulphate.

11. A method according to claim 7, 8 or 9 wherein the substrate is an ester and the enzyme is an esterase.

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